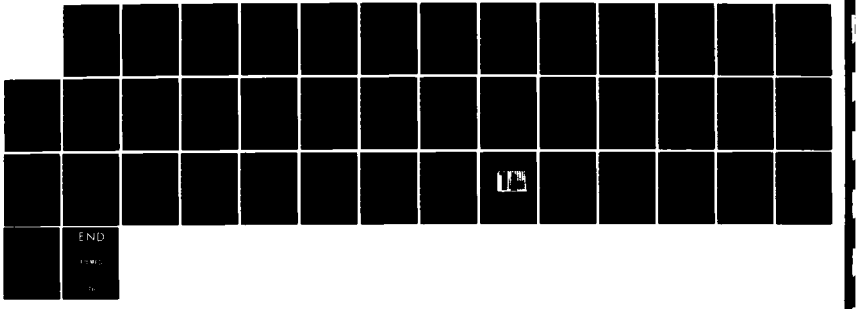
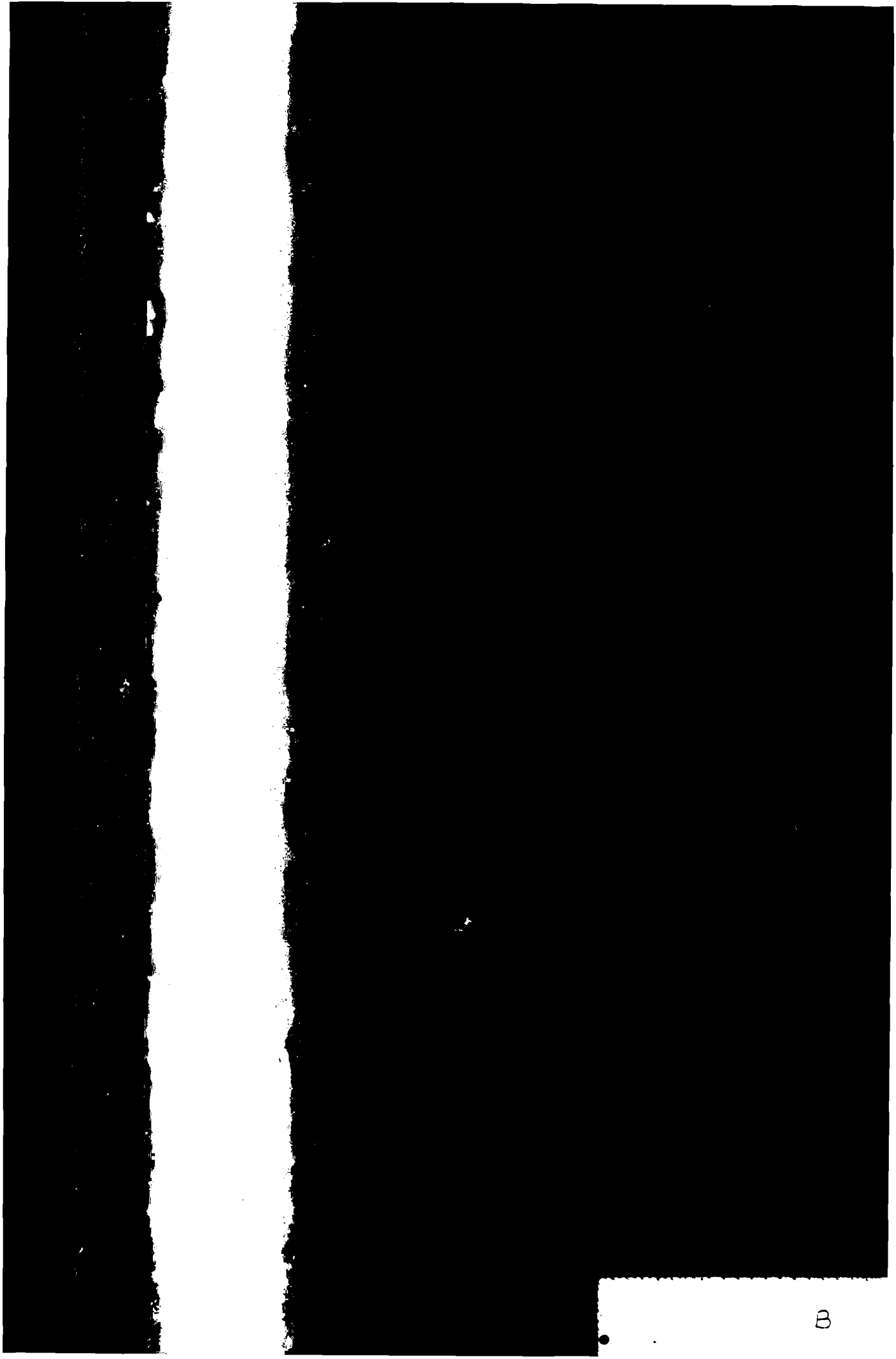
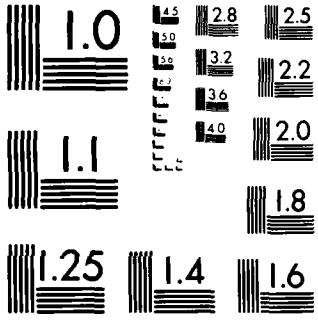
AD-A149 822 BIOCHEMISTRY OF TRYPANOSOMATIDAE OF IMPORTANCE IN AFRICA(U) MASSACHUSETTS UNIV AMHERST DEPT OF MICROBIOLOGY L L NOLAN DEC 82 DAMD17-81-C-1198 1/1

UNCLASSIFIED F/G 6/1 NL





B



AD_____

Biochemistry of Trypanosomatidae of Importance in Africa

Annual Report

Linda L. Nolan, Ph.D.

December 1982

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1198

University of Massachusetts
Amherst, Massachusetts 01003

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A149822
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Biochemistry of Trypanosomatidae of Importance in Africa	5. TYPE OF REPORT & PERIOD COVERED Annual Report (1 Jan. 1982 - 31 Dec. 1982)
6. PERFORMING ORG. REPORT NUMBER	
7. AUTHOR(s) Linda L. Nolan, Ph.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-81-C-1198
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology University of Massachusetts Amherst, MA 01003	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AF.080
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command; Fort Detrick, Frederick, MD 21701-5012	12. REPORT DATE December 1982
	13. NUMBER OF PAGES 42
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Leishmania/mode of action of formycin B and metabolites/purine and pyrimidine transport and metabolism/chemotherapy.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to synthesize purines de novo and consequently are obligate salvagers of preformed purines.

This project involves an investigation of purine and pyrimidine metabolism of Leishmania. Comparisons of their biochemistry will be made with in the

parasitic group and to their host cells.

Basic information regarding metabolic capacities of these organisms will be obtained. Attention will be given to the mechanisms by which these organisms absorb nutrients from their environment. These mechanisms involve enzymes excreted into their surroundings, enzymes located on the cell surface, and enzymes located within the cell.

Enzyme and transport mechanisms which exhibit differences from those of host cells will offer targets for chemotherapeutic exploitation. Inhibitors will be sought which will affect these target systems. Those inhibitors which are leichmanicides will then be tested in an appropriate animal system.

During this year uptake capabilities of L. donovani (Khartoum strain-drug sensitive visceral leishmaniasis) and L. braziliensis panamensis WR 227 were investigated. In L. donovani WR 130 it was found that N^6 methylamino-purine inhibited uptake of hypoxanthine, guanine and to a lesser extent adenine. 6-Methylaminopurine 9-ribofuranoside inhibited uptake of adenosine and to a lesser extent guanosine.

In L. braziliensis WR 227 it was determined that at least two loci exist for the transport of nucleosides, one for adenosine and one for inosine and guanosine.

Uptake experiments using ^{3}H-formycin B, showed that its uptake is inhibited by inosine and guanosine, but not by adenosine.

The following enzymes in L. braziliensis were tested for inhibition by formycin B (2 μM - 1 mM) with no significant effect observed.

- adenine, guanine and hypoxanthine phosphoribosyltransferases
- Guanase
- Adenase
- Inosine, guanosine and adenosine nucleosidases

Preliminary studies have shown that allopurinolriboside and formycin B appear to be effective analogues against certain species of leishmania and trypanosomes. The biochemical mode of action of these compounds appears to be similar but to vary quantitatively. Our preliminary results suggest that the most critical action of these drugs occurs by there interaction with RNA and DNA. Investigations will be made on the mode of action these compounds have on the RNA and DNA of sensitive organisms, and how this process can be reversed by the addition of other purines.

Other purine and pyrimidine analogues found to be inhibitory to leishmania will be investigated for their effect on interference with nucleic acids and their metabolism.

AD_____

Biochemistry of Trypanosomatidae of Importance in Africa

Annual Report

Linda L. Nolan, Ph.D.

December 1982

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1198

University of Massachusetts
Amherst, Massachusetts 01003

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

SUMMARY

A comparison of the enzymes of pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. The enzymes involved in purine salvage are of particular interest because most pathogenic protozoa lack the ability to snythesize purines de novo and consequently are obligate salvagers of preformed purines.

This project involves an investigation of purine and pyrimidine metabolism of Leishmania. Comparisons of their biochemistry will be made within the parasitic group and to their host cells.

Basic information regarding metabolic capacities of these organisms will be obtained. Attention will be given to the mechanisms by which these organisms absorb nutrients from their environment. These mechanisms involve enzymes excreted into their surroundings, enzymes located on the cell surface, and enzymes located within the cell.

Enzyme and transport mechanisms which exhibit differences from those of host cells will offer targets for chemotherapeutic exploitation. Inhibitors will be sought which will affect these target systems. Those inhibitors which are leishmanicides will then be tested in an appropriate animal system.

During this year uptake capabilities of L. donovani (Khartoum strain-drug sensitive visceral leishmaniasis) and L. braziliensis panamensis WR 227 were investigated. In L. donovani WR 130 it was found that N^6 Methylaminopurine inhibited uptake of hypoxanthine, guanine and to a lesser extent adenine. 6-Methylaminopurine 9-ribofuranoside inhibited uptake of adenosine and to a lesser extent guanosine.

In L. braziliensis WR 227 it was determined that at least two loci exist for the transport of nucleosides, one for adenosine and one for inosine and guanosine.

Uptake experiments using ^{3}H-formycin B, showed that its uptake is inhibited by inosine and guanosine, but not be adenosine.

The following enzymes in L. braziliensis were tested for inhibition by formycin B (2 µM - 1 mM) with no significant effect observed.

- adenine, guanine and hypoxanthine phosphoribosyltransferases
- Guanase
- Adenase
- Inosine, guanosine and adenosine nucleosidases

Preliminary studies have shown that allopurinolriboside and formycin B appear to be effective analogues against certain species of leishmania and trypanosomes. The biochemical mode of action of these compounds appears to be similar but to vary quantitatively. Our preliminary results suggest that the most critical action of these drugs occurs by their interaction with RNA and DNA. Investigations will be made on the mode of action these compounds have on the RNA & DNA of sensitive organisms, and how this process can be reversed by the addition of other purines.

Other purine and pyrimidine analogues found to be inhibitory to leishmania will be investigated for their effect on interference with nucleic acids and their metabolism.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Routine Methods: more specific procedures currently being used in our laboratory are discussed in *Resume* of Progress to date.

Culture Methods:

The organisms used in this project have been obtained from the Walter Reed Army Institute of Research through the curtesy of Dr. Joan Decker-Jackson and Dr. Jonathan Berman. The organisms used most have been Leishmania braziliensis panamensis WR 227 and L. donovani WR 130 (Khartoum strain-drug sensitive visceral leishmaniasis). Other organisms presently being cultivated in this laboratory are L. braziliensis WR 424 (Murray isolate from Panama causing cutaneous leishmaniasis), L. braziliensis WR 063 (Terborgh isolate from Peru, causing mucocutaneous leishmaniasis). These organisms are maintained by weekly transfers into Schneider's medium [Grand Island Biological Co., Grand Island, N.Y. (Gibco)] containing 10% heat inactivated fetal bovine serum (HIFBS: GIBCO).

For growing large batches of leishmaniae promastigotes, Brain Heart Infusion Medium (BHI) containing 37 g Difco Brain Heart Infusion/liter water, 10% heat inactivated serum and 26 µg hemin/ml is used. Cells are grown at 26°C in 2000 ml wide Fernbach flasks containing 250 ml of BHI and harvested during the exponential growth phase (∿ day 4).

For defined biochemical experiments we use the media of Steiger and Black. We use this media for all transport, uptake and reversal experiments. To deplete cells of purines we transfer an inoculum from Brain Heart Infusion into Steiger and Black minus purine, but with 5% serum, and incubate these cells 48 hr at 26°C. We then aseptically centrifuge these cells 5000 xg for 10 min. and re-suspend them into fresh Steiger and Black minus purine. We resuspend to the desired number of cells and then add the purine or analogue we wish to test. By treating the cells in this manner, we avoid as much as possible interference of the metabolism of the compound being tested by purines in the media.

RE 1X (Steiger and Black)

Components per liter:

A)	8.0g	NaCl	C)	300 mg	L-glutamine
	400 mg	KCl		1.0 g	$NaHCO_3$
	200 mg	$MgSO_4 \cdot 7H_2O$		14.25 g	HEPES (=60 mM)
	60 mg	$Na_2HPO_4 \cdot 2H_2O$		20 mg	adenosine
	60 mg	KH_2PO_4			
	2.0g	glucose	D)	1 mg	D-biotin
				1 mg	choline chloride
B)	200 mg	L-arginine		1 mg	folic acid
	100 mg	L-histidine		2 mg	i-inositol
	100 mg	L-isoleucine		1 mg	niacinamide
	300 mg	L-leucine		1 mg	D-pantothenic acid (hemi-calcium salt)
	250 mg	L-lysine. HCl			
	50 mg	L-methionine		1 mg	pyridoxal·HCl
	100 mg	L-phenylalanine		0.1 mg	riboflavine
	300 mg	L-proline		1 mg	thiamine.HCl
	400 mg	L-threonine			
	50 mg	L-tryptophan	E)	2.5 mg	haemin
	50 mg	L-tyrosine			
	100 mg	L-valine			

L. donovani will be maintained in male Syrian hamsters by intraperitoneal injection of infected hamster spleen homogenate. Amistigotes will be isolated by sterile removal of infected spleens which are then homogenized and the parasites cleaned by differential centrifugation (Krassner, 1966).

Enzyme Studies

In all cases where it becomes critical to resort to enzyme purifications the sequence of steps envisioned are gel filtration, isoelectrofocusing, column chromatography, gel electrophoresis, various pH changes and heat treatments. Activities will be largely determined by the use of radioactive substrates. When possible, commercial mammalian enzyme preparations will be used to compare with enzymes being studied in the protozoans; otherwise, mammalian (bovine and/or rat) liver will be used as a source of tissue.

Enzyme activities are determined by paper chromatography or by paper electrophoresis as has been previously described (Kidder, Dewey and Nolan, 1977), following incubations of reactants with [^{14}C]-labeled substrate. When inhibitors are used they are added to the enzyme-buffer solution, at optimum pH, and preincubated 10 min at 35^{o}. After reaching equilibrium at 35^{o} the radioactive substrate is added. The reaction is stopped at the appropriate time and the reaction mixture is streaked on Whatman #1 paper for descending chromatography or for electrophoretic separation. Radioactive peaks are located with the aid of a Tracerlab 4π scanner and identified by co-chromatographs of authentic samples. Quentitation is accomplished by determining the areas under the peaks by planimetry. Some enzyme activities are determined using the HPLC equipped with a scintillation counter.

In those cases where radioactive substrates are not commercially available, if possibly they will be synthesized enzymatically from an available radioactive precursor. Spectrophotometric assays will be carried out if a radioactive assay is not feasible. In general the assay methods that will be used, are those described in Methods of Enzymology LI, Purine and Pyrimidine Metabolism, 1978.

Transport and Accumulation Studies

These studies are carried out using whole cells of the parasites along with labeled purine and pyrimidine bases and nucleosides. Incubations are carried out in 1.5 ml microcentrifuge tubes in the presence and absence of substrates and/or compounds being tested for inhibition.

By transport is meant the events essential to translocation of the substrate across the cytoplasmic membrane. As a consequence of this translocation, substrates may become available to intracellular enzymes and metabolized to chemical forms that are not substrates for the transport systems. The accumulation of such metabolites (as well as of the chemically unaltered substrate) is properly referred to an uptake. Uptake is several steps removed from transport. The availability of energy, which may determine the concentration of substrates against electrochemical gradients and the activities of intracellular enzymes which affect their metabolism, may limit uptake but are not directly related to the transport event (Berlin and Oliver, 1975).

The most serious problem in studying the process of transport as distinct from uptake is the failure to determine rates at sufficiently early times. As with enzyme reactions, it is essential to measure initial rates in order to determine unidirectional flux. This period is clearly incompatible with prolonged washings or centrifugations, and rapid sampling methods must be employed. A technique was devised by Kidder, Dewey and Nolan (1978) which facilitates rapid measurement. This procedure is based on rapid separation of medium from cells by centrifugation. Aliquots of cell suspension are placed in plastic centrifuge tubes (1.5 ml capacity), radioactive substrate added, vortexed briefly and the tubes placed in an Eppendorf microcentrifuge, and at 5 sec. the cells are rapidly sedimented. Aliquots are placed on planchets for counting. The cells are then resuspended by vigorous vortexing and two further samples are plated to obtain total (zero time) counts. The presence of cells in the latter samples necessitate a correction for self adsorption. The difference between the counts represents the amount of substrate taken in by the cells.

The factor used for correction of self adsorption by the whole cells is obtained by incubating cells with AMP (p^{32} labels, which does not enter C. fasciculata cells) spining them down and taking aliquots of the supernant, then resuspending the pellet by vortexing and determining radioactivities. The difference between counts obtained with supernant plus cells and supernant is taken as self absorption. Thirty duplicate experiments are conducted and an average taken.

This technique that we have used for C. fasciculata has been successfully used in investigating uptake of purine bases and nucleosides in African trypanosomes (James and Born, 1980).

Transport and uptake experiments dealing with macrophage and tissue culture cells will be by the method of Berlin and Oliver (1975).

Protein

Protein will be estimated by methods II, III or IV of Layne (1957) or by the method of Kalb and Vernlohr (1975), depending on the amount of protein and nucleic acids in the sample.

Inhibitor Studies

Most inhibitors selected for testing will be structural analogs of the enzymes or transport systems substrate or product. Compounds which appear as likely candidates include the following:

Formycin B
Formycin-B-monophosphate
Formycin-A-monophosphate
Formycin-A-diphosphate
Formycin-A-triphosphate
Allopurinol
Allopurinolriboside
4-amino-(3,4-d)pyrazolopyrimidine
6-4,T-Dimethylallylaminopurine

phenylhydrazinopyrimidine
hydroxyphenylazopyrimidine
2-Aminopyrimidine
N6-Methyladenine
N6-Dimenthyladenine

6-Mercaptopurine
6-Methylmercaptopurine
6-Chloropurine
2-Methyladenine
1-Deazaadenine
Purine
2,6-Diaminopurine
2-Azaadenine
2-Aminopurine

Isoguanine
8-azaguanine

2-Hydroxypurine
8-Methyladenine
2-Methylthio-6-aminopurine
Kinetin
N1-Methylhypoxanthine
6-Methyl-2-oxypurine
6-Phenylaminopurine
6-Benzylaminopurine
6-Bromopurine
6-Methoxypurine

coformycin
deoxycoformycin
erythro-9-(2-hydroxy-3-nonyl) adenine
6-mercapto-9-(tetrahydro-2-furyl)-purine
2,6-bis-(hydroxyamino)-9-B-D-ribofuranosyl-purine
6-iodo-9-(tetrahydro-2-furyl) purine
Xylosyladenine
5-methylrotate
isooorotic acid
4,2-substituted, oxazolo-[5,4-d]-pyrimidine -7-one
3-amino-4-carbethoxypyrazole
3-amino-4-carboxypyrazole

N^6-benzylaminopurine
N^6-(2-naphthylamino) purine
N^6-(2-thenylamino) purine
N^6-(2-pyridylamino) purine
5-cyanouracil
5-iodouracil
5-fluorocytosine
Azapyrimidines
5-fluorouracil
6-aminothymine
dihydroazaorotate
5-nitroorotate

isoorotic acid

In the event that derivatives of formycin are synthesized by Dr. Roland K. Robins, Director of the Cancer Research Center in Brovo, Utah, these will be tested for growth inhibition and if effective their mode of action will be determined at the membrane, enzyme and RNA & DNA level. Dr. R.K. Robins is under contract with WRAIR.

Phase Contract Photography

Compounds which affect purine or pyrimidine metabolism will be investigated to see what effect they have on cellular morphology. These studies will be carried out with the use of a Zeiss phase contrast microscope equipped with a polaroid land camera.

Metabolic Fate of Inhibitors

Spectroscopic and chromatographic methods will be used to determine the metabolic fate of inhibitors.

Most of the metabolism studies will be performed using our Waters HPLC system equiped with a UV-detector and an on-line scintillation counter (HS model) purchased from Radiomatic Instruments. Using this system we are able to use dual labeling, which allows us to follow the radioactive fate of two radioistopes at one time. Depending on the type of separation (anion-exchange or reversed-phase), the system measures simultaneously the concentration and radioactivity for a given chromatographic peak. Rapid and reproducible results may be obtained and recorded as both analog (peak trace) and digital output (peak integration). Through the use of purine and pyrimidine precursors, it is possible to study in a systematic way the overall metabolism of nucleotides, nucleosides and bases.

Our system is similar to that of H. Kyle Webster's at WRAIR.

At the present time we are using a radial-pak μ Bondapak C-18 cartridge with a Z-module system (Waters Assoc., Milford, MA) for separation of purine bases and nucleosides. An isociatic solvent of 50% methanol: H_2O is being used to separate most bases and nucleosides, but to separate formycin A from formycin B we use 10% methanol: H_2O at a flow rate of 3 ml/min. (Fig.). To separate nucleosides and nucleotides, we use a Waters Radial-Pak SAX cartridge with the Z-module using a gradient of KH_2PO_4:KCl and varying flow rates (see Fig. A, B). As regards the analysis of formycin B and its metabolites we are trying the conditions reported by Rainey & Santi (PNAS, 1983, February issue) and of Rossomando, et al (Anal. Biochem. 116, 80-88, 1981). The procedure used by Rossomando takes advantage of the fluorescent properties of the formycin derivatives which is much more sensitive than UV-detection. He also uses a reverse-phase isocratic system for separation of formycin nucleotides which is quicker and has less UV background noise than the conditions reported by Rainey & Santi.

Preparation of cell extracts are done by the method of Pogolotti & Santi (Anal. Biochem. 126, 334-345, 1982). Briefly, this method involves 0.6MTCA extraction and centrifugation at 4°C and the supernant is aspirated and added to an equal volume of cold freon containing 0.5 M tri-n-octylamine. The mixture is vortexed and centrifuged (12,000 g, 30 sec.) at 4°C, the lower phase is removed by aspiration leaving the near neutral aqueous solution of nucleotides. These extracts are then stored at -70°C if not used immediately.

A) 10% Methanol-H_2O Isocratic Elution

Time	Flow	% A	% B	Curve
Initial	3	100	0	*

chart speed: 0.5 cm/min

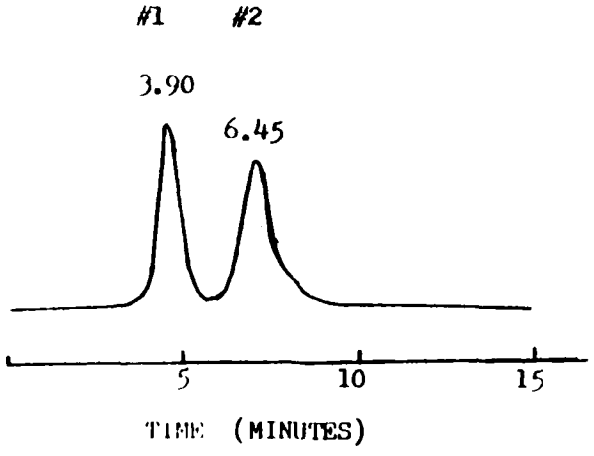
column: Radial-PAK μBONDAPAK C-18 Cartridge, 8mm X 10 cm with Z-Module System

peaks: #1 Formycin B
#2 Formycin A

sample: 0.5 mM Formycin A
0.5 mM Formycin B

absorbance: 254 nm

sensitivity: 0.05



A) 0.007:0.007 KH_2PO_4:KCL, pH 4.0
B) 0.25:0.5 KH_2PO_4:KCL, pH 5.0

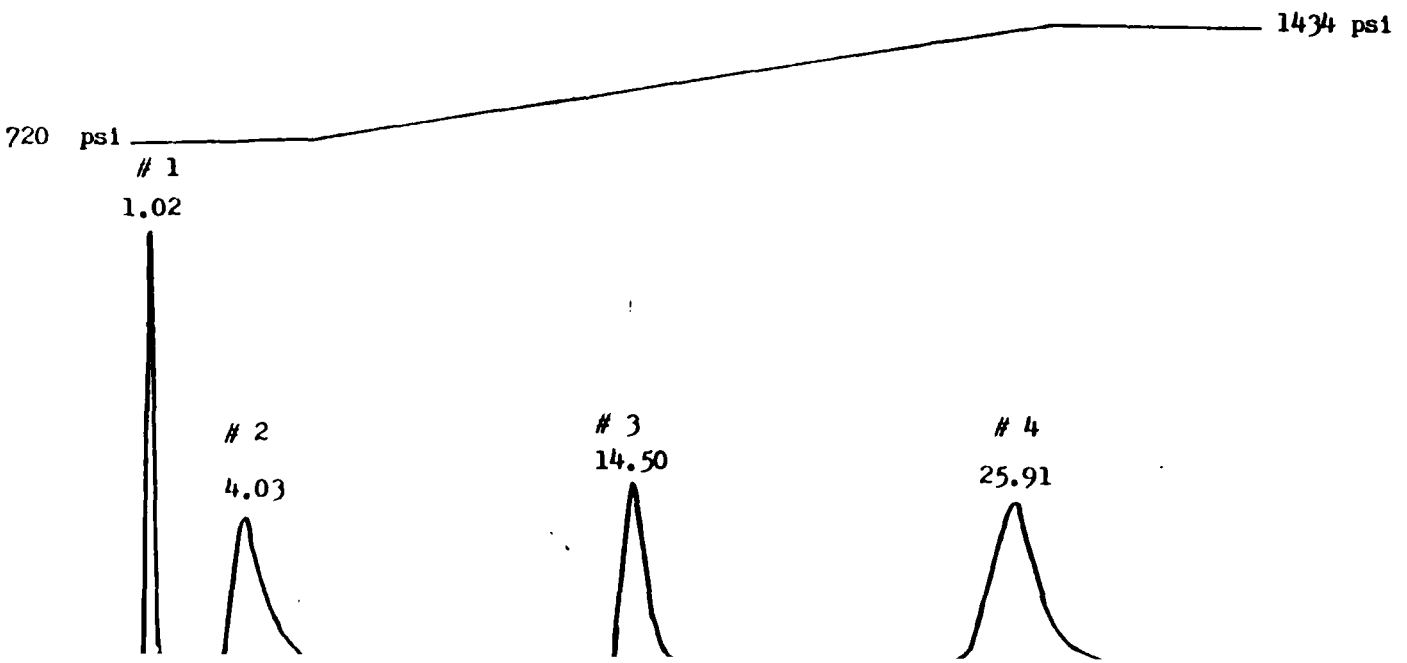
Time	Flow	%A	%B	Curve
Initial	3	100	0	*
6	3	100	0	6
26	5	0	100	6
35	5	0	100	6

chart speed 0.5 cm/min

column: Radial-PAK SAX Cartridge, 10 μ 8mm X 10 cm w/ Z-module system

absorbance: 254 nm sensitivity: 0.05

peaks: #1 Formycin A
#2 Formycin A monophosphate
#3 Formycin A diphosphate
#4 Formycin A triphosphate



SPECIFIC AIMS and MILITARY SIGNIFICANCE:

The need for leishmanicides cannot be overemphasized. At present chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality, presumably due to strain differences in Leishmania.

To date the logical design of antiparasitic drugs has proved largely unsuccessful with the exception of purine metabolism in protozoa. While mammalian cells are capable of de novo syntbesis of purines, many parasites do not synthesize purines but use salvage pathways. Analogues inhibiting key enzymes in the salvage pathways should, therefore, provide novel therapeutic agents. Purines and pyrimidines serve not only as precursors of RNA and DNA, but also as stores of high energy phosphate, constituents of certain coenzymes, and modulators of various enzymatic reactions. In view of this vital role, intervention of their metabolism will have profound effects on the organism.

To date there is no safe, effective, and quality-controlled antiparasitic vaccines. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult.

Parasite enzymes located on or near the surface of the membrane and transport and receptor proteins on the membrane which differ from the hosts are candidates as molecules which provoke host-protective immunity.

Leishmania and Trypanosoma studied have been found to require exogenous purine (Gutteridge, 1978) as opposed to the majority of mammalian cells. This fact together with the finding that some purine metabolizing enzymes are different (Kidder & Nolan 1979: Nolan & Kidder, 1980) may provide a useful target for immune intervention.

The elucidation of the biochemical mode of action of promising compounds (allopurinol riboside, formycin B) will permit the logical design of more effective derivatives and also will provide insight on the mechanism of drug resistance. This information may allow a therapy program to be developed which would decrease or eliminate the problem of drug resistance.

SPECIFIC AIMS

1) Transport and uptake of purine and pyrimidine bases and nucleosides will be determined in L. braziliensis and L. donovani. A continued search for inhibitors of these processes will be carried out, and these compounds will be tested for growth inhibition.

2) Inhibitors will be sought for key enzymes or unique enzymes in the purine salvage pathway and the pyrimidine de novo pathway.

From Figure 1 it can be seen that a block in early purine salvage metabolism will not lead to a complete shutdown of purine metabolism. Bases and nucleosides can be interconverted by pivotal enzymes which convert adenine nucleotides to guanine nucleotides and vice versa. Inhibitors of the enzymes (Nos. 9, 10, 11, 14, 15 and 17 in Figure 1 (should provide effective blocks in metabolism so that host purine sources cannot be converted to essential leishmanial nucleotides. We will characterize these enzymes and search for inhibitors.

Leishmania contain unique enzymes involved in pyrimidine metabolism (See Background) namely, dihydroorotate hydroxylase, oritidine-5'-phosphate hydroxylase and orotate phosphoribosyltransferase. Inhibitors will be sought for these compounds.

3) The mode of action of formycin B and its major metabolites, formycin A mono, di & triphosphate will be determined at the transcription and translation level. Their mode of action will be compared to allopurinol riboside and 4-aminopyrazolopyrimidine.

Altered structure of mRNA, tRNA and DNA will be determined by in vitro assay techniques and gel electrophoresis. RNA and DNA polymerase will be purified and formycin compounds will be tested for inhibition of their activity.

Once we determine the site or sites of action of formycin metabolites, if WRAIR wishes we will test formycin derivatives being synthesized by Dr. Roland K. Robins, Director of the Cancer Research Center, Brovo, Utah. Dr. Robins is also under contract to WRAIR. Techniques which will be employed are described in: 1). Clemens, et al., J. Biol. Chem. 250, 2 (1974) 522-526; 2). Methods in Enzymology, Vols. XX, XXI, XXII. These volumes describe techniques for nucleic acid and protein synthesis studies and isolation of RNA and DNA polymerases.

4) The organisms which will be used in our studies will be L. braziliensis panamensis WR 227 and L. Donovani (Khartoum strain drug sensitive visceral leishmaniasis WR 130. If WRAIR would like us to investigate other organisms, we will do so.

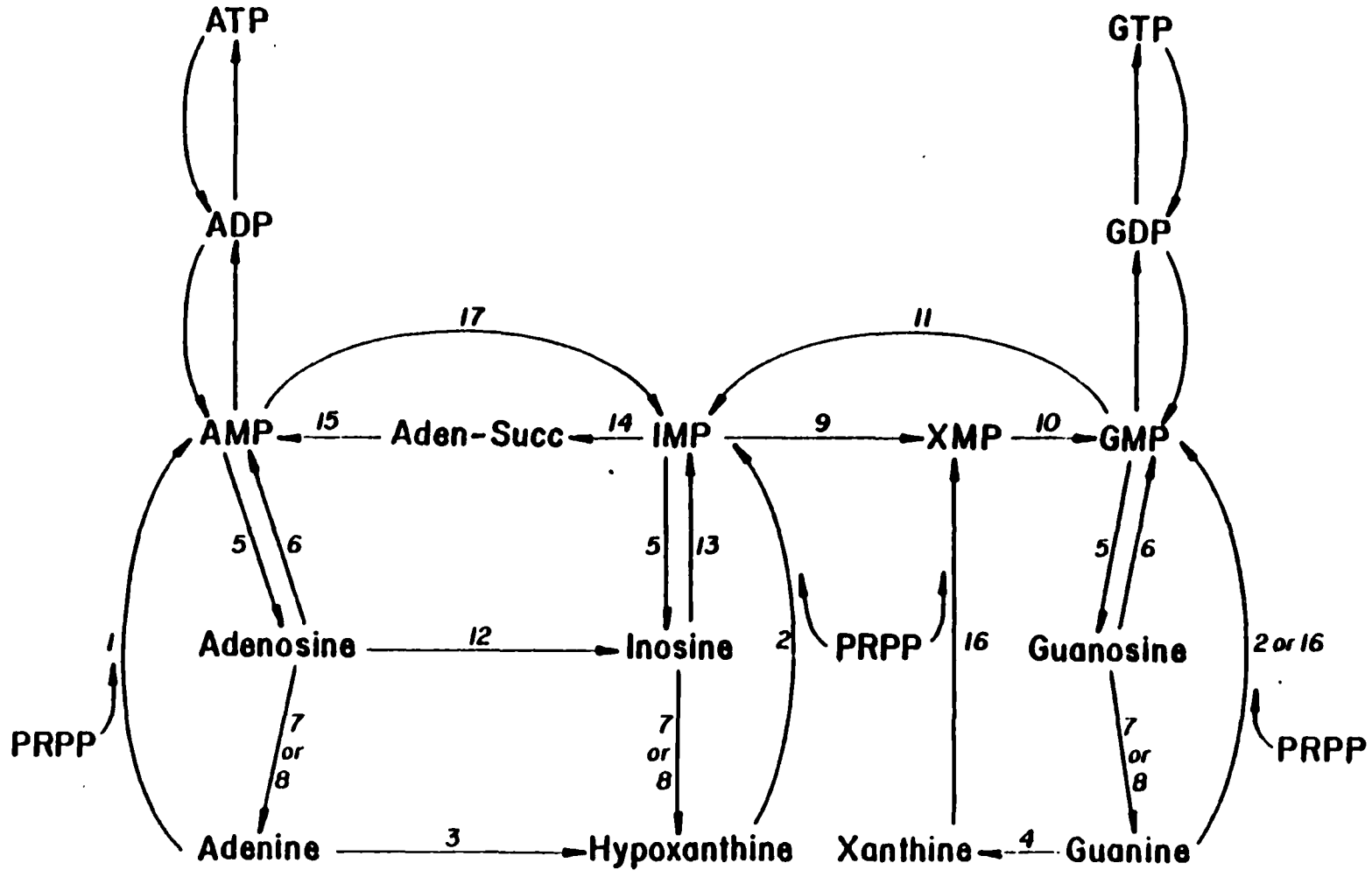

ATP
GTP
ADP
GDP
17
11
AMP
15
Aden-Succ
14
IMP
9
XMP
10
GMP
5
6
5
13
5
6
1
Adenosine
12
Inosine
2
PRPP
16
Guanosine
2 or 16
PRPP
7
or
8
7
or
8
7
or
8
PRPP
Adenine
3
Hypoxanthine
Xanthine
4
Guanine

FIGURE 1

Figure 1. Some of the reactions involved in interconversions of purines and purine derivatives. This is a compendium of reactions present in various organisms.

Enzymes: 1, adenine phosphoribosyltransferase (EC 2.4.2.7); 2, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); 3, adenine deaminase (EC 3.5.4.2); 4, guanine deaminase (EC 3.5.4.3); 5, 5'-nucleotidase (EC 3.1.3.5); 6, adenosine kinase (EC 2.7.1.20); 7, purine nucleoside hydrolase (EC 3.2.2.1); 8, purine nucleoside phosphorylase (EC 2.4.2.1); 9, IMP dehydrogenase (EC 1.2.1.14); 10, GMP synthetase (EC 6.3.4.1); 11, GMP reductase (EC 1.6.6.8); 12, adenosine deaminase (EC 3.5.4.4); 13, inosine kinase (EC 2.7.1.73); 14, adenylosuccinate synthetase (EC 6.3.4.4); 15, adenylosuccinate lyase (EC 4.3.2.2); 16, guanine phosphoribosyltransferase (bacterial); 17, AMP deaminase (EC 3.5.4.6).

Abbreviations: Ad, adenine, Hx, hypoxanthine; X, xanthine; Gu, guanine; AdR, adenosine; In, inosine, GuR, guanosine; S-AMP, adenylosuccinate; AMP, adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; IMP, inosine 5'-monophosphate; XMP, xanthosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate.

RESUME of PROGRESS TO DATE - Results and Discussion

Efforts during this year have centered on the following areas:

- Characterization of purine and pyrimidine transport and uptake capabilities, and testing of analogues to inhibit these processes.
- The testing of purine and pyrimidine analogues in growth experiments using a biochemically defined medium.
- The setting up of a high performance liquid chromatography system equipped with a UV-detector and an on-line scintillation counter. Personnel have been trained at the Water's HPLC school in Milford, MA. Techniques for the separation of purine and pyrimidines are being tested to determine which ones best suit our needs.
- The metabolism and mode of action of formycin B is being investigated and being compared to that of allopurinolriboside and 4-aminopyrazolopyrimidine at the (a) growth level, (b) enzymatic level and (c) at the level of translation.

Research during the next year will be a continuation of all the above with the major effort in comparing the mode of action of formycin B to that of allopurinolriboside and 4-aminopyrazolo(3,4-d)pyrimidine at the enzymatic level and at the level of translation and transcription.

$$\text{DNA} \xrightarrow[\text{transcription}]{} \text{RNA} \xrightarrow[\text{translation}]{} \text{protein}$$

Efforts during the first three month period of this year have dealt with: (1) Cultivation of Leishmania braziliensis WR 424 (Murray isolate from Panama) (cutaneous leishmaniasis), L. braziliensis WR 063 (Terborgh isolate from Peru, mucocutaneous leishmaniasis), and L. donovani WR 130 (Khartoum strain-drug sensitive visceral leishmaniasis) in a medium minus blood, so that transport and enzymatic experimentation can be carried out. Presently, L. braziliensis WR 424 and WR 063 are being cultivated in 70% Brain Heart Infusion, 30% fetal calf serum or 70% Medium 199 and 30% fetal calf serum (v/v). Leishmania donovani WR 130 can be cultivated in the medium of Steiger and Black (no serum or blood) if transferred from Brain Heart Infusion medium plus 10% fetal calf serum. Cells are being grown, centrifuged, washed, sonicated and then frozen for future enzymatic work. (2) Transport and uptake experiments were investigated in L. donovani WR 130 since it is easiest to grow in a defined medium and because of its importance to the Army (Hendricks and Jackson, personal communication). Uptake experiments were performed as described by Kidder, Dewey and Nolan, 1978, J. Cell Physiol. 96, 165-170. Cells were initially grown in Brain Heart Infusion plus 10% fetal calf serum for 3 days and then 30 ml of cells and medium were transferred to Steiger and Black medium (250 ml) minus purine. The cells were incubated 24 hrs. to deplete them of purine pools, harvested and resuspended in Steiger and Black medium minus purine (1×10^6 cells/ml). Purine analogs (inhibitors) were added 5 min. before adding C^{14} purines. Aliquots were taken 10 - 120 min. and C^{14} purine

uptake was determined. Table I gives concentrations used, purine and purine analogs and two representative times. N^6 methylaminopurine and 6-methylaminopurine 9-ribofuranoside were found to inhibit uptake of various purines.

Caffeine and theophylline (methylated purines) at 1 mM did not inhibit uptake of any purines.

TABLE:1 Uptake of Purines and Their Inhibition in L. donovani WR 130

Purine nM	Purine analogue Inhibitor		% Inhibition
Adenine (.11 mM)	N^6methylaminopurine (.55 mM)	15 min	38.7
		60 min	59.6
Hypoxanthine (.11 mM)	N^6methylaminopurine (.55 mM)	15 min	100
		60 min	100
Guanine (.011 mM)	N^6methylaminopurine (.55 mM)	15 min	97
		60 min	100
Guanosine (.11 mM)	6-methylaminopurine 9-ribofuranoside	60 min	41.3
		120 min	67.5
Adenosine (.11 mM)	6-methylaminopurine 9-ribofuranoside (.55 mM)	60 min	100
		120 min	100

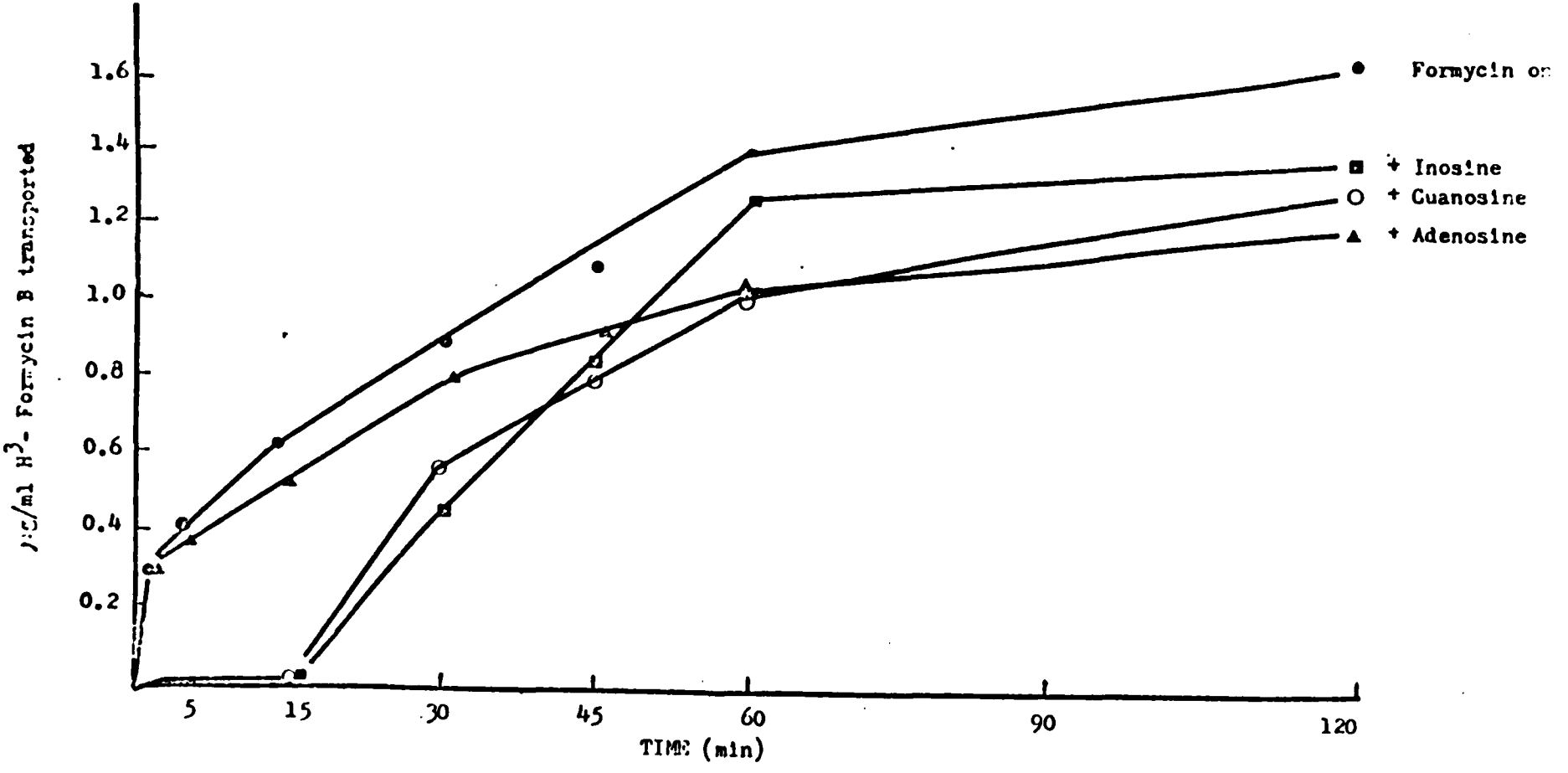
Research During Second Three Month Period

Research during this period has centered on the mode of action of Formycin B in Leishmania WR 227. Uptake and incorporation studies of purine bases and nucleosides were observed in the presence and absence of Fromycin B. H^3 Formycin B uptake was observed alone and in the presence of other purines. Purine salvage enzymes were tested for inhibition by Formycin B. H^3 Formycin B was incubated with whole cells which were then disrupted and the products of Formycin B determined. Assays were carried out to determine if Formycin B (or product) was phosphorylated by a purine phosphoribosyltransferase or by a kinase.

Fig.2 , shows uptake of H^3-Formycin B (2 ug/ml) in the presence of purine nucleosides (200 ug/ml) in the defined media of Steiger and Black. As shown, from 1-15 minutes inosine and guanosine completely inhibited H^3 Formycin B uptake. Adenosine during this time period shows little effect, but inhibits transport more than the other nucleosides after 60 minutes. At the end of 120 minutes (Fig 2) adenosine inhibits uptake of Formycin B 18.42%, guanosine 11.18% and inosine 10.55%.

Fig.2

Transport of H^3- Formycin B in the Presence of Purine Nucleosides



It appears likely that there are two transport proteins that Formycin B uses to enter cells. One a rapid high affinity system (as observed 1-15 min range) which in the presence of other purine nucleosides inhibits uptake of Formycin B. Uptake of Formycin B in the presence of other nucleosides is only depressed 11-18% by the slower transport system.

Uptake of C^{14} Inosine and Guanosine in the presence of different concentrations of Formycin B is shown in Fig.3 . Data was obtained between 1-120 minutes, but only the 120 minute data is shown. Formycin B (at 200 µm) inhibited uptake of guanosine 24% and adenosine 12%.

To determine the concentration of Formycin B concentrated within cells and its rate of uptake, H^3-Formycin B (0.5 µg/ml in the media used by Berman, Schneider's plus S-HOMEN at a cellular concentration of 6.5×10^8/ml) was assayed for uptake. As shown in Fig.4 , uptake was linear up to 4 hrs. and then leveled off. It was determined by volume displacement that Formycin B (0.5 µg/ml) was concentrated 6.42 times inside the cells giving it a concentration of 3.21 µg/ml packed cells.

The following enzymes were tested for inhibition by Formycin B (2 µM-1mM) with no significant effect observed.

1) adenine, guanine and hypoxanthine phosphoribosyltransferases
2) Guanase
3) Adenase
4) Inosine, Guanosine and Adenosine nucleosidases

A great deal of effort has been spent trying to assay Inosine, guanosine and adenosine kinase activity. These enzymes are extremely low in activity or very labile. Also the nucleoside hydrolases compete for the substrate making it difficult to assay only kinase activity. Nucleoside kinases in WR 227 are not a significant system and not the major mode of action of Formycin B.

We found that Formycin B is not phosphorylated via the purine phosphoribosyltransferases or by a nucleoside kinase. It appears that the first enzyme to act on Formycin B is the nucleosidephosphotransferase as first reported by Carson & Chang (Biochem. Biophys. Res. Comm. 100, 1377-1383, 1981).

Research During Third Three-Month Period

Efforts during this period have centered on setting up a fully automated high performance liquid chromotography system (HPLC) for assay of purine bases, nucleosides and their analogues. We have obtained a Waters analytical HPLC system with a microprocessor and autosampler. We are in the process of determining the best method of separation of purine compounds for our particular purposes.

Research during this period has centered on the mode of action of Formycin B in Leishmania WR 227. In order to determine the effect of purine bases and nucleosides on Formycin B inhibition, WR 227 was grown in the medium of Steiger

Fig.3 Uptake of Purine Nucleosides in the Presence of Formycin B

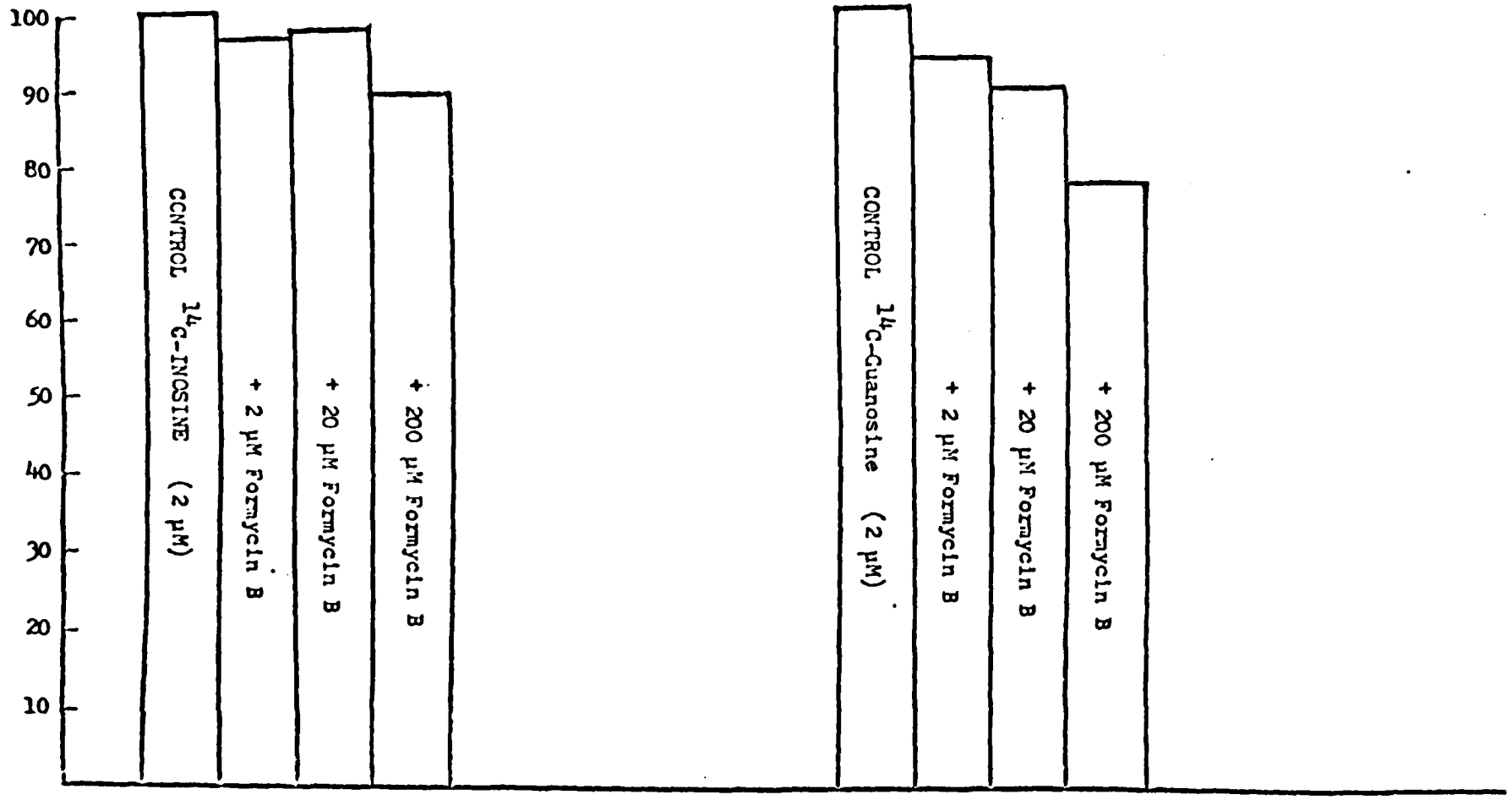
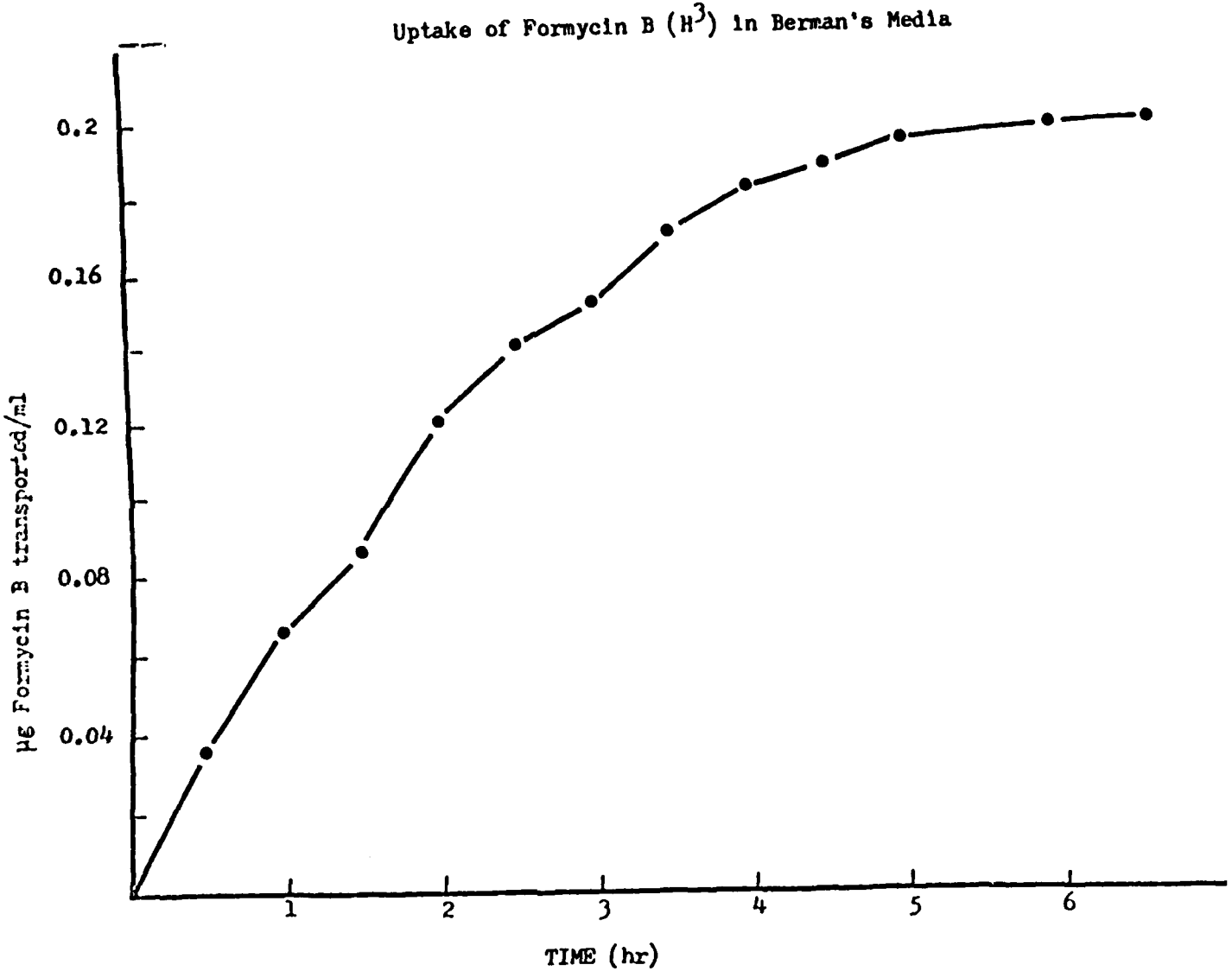


Fig. 4


Uptake of Formycin B (H^3) in Berman's Media
µg Formycin B transported/ml
0.2
0.16
0.12
0.08
0.04
1
2
3
4
5
6
TIME (hr)

and Black minus purine. Different purines were added at different concentrations to determine if they could release inhibition by Formycin B. Formycin B concentrations remained constant at 5 μM with purine concentrations at 0.02 mM, 0.1 mM and 1.0 mM. As seen in Fig.5 , inosine at the concentrations used was the most efficient purine to reverse the effects of Formycin B, followed by guanosine and adenosine. Increasing adenine, guanine and hypoxanthine concentrations did not reverse growth inhibition by Formycin B. From previous work we know that nucleosides in WR 227 are rapidly broken down to the bases by nucleosidases. Because of this, one would expect that nucleosides would have the same effect as bases. The possibility exists that as nucleoside concentrations increase, they are converted to monophosphates via nucleoside phosphotransferase. By increasing nucleoside concentrations competition would exist with Formycin B for this enzyme. In this situation less Formycin B would become phosphorylated and presumably less toxic. Currently nucleoside phosphotransferase is being partially isolated to perform kinetic studies to confirm this possibility. Cells grown in the presence of different purines will be examined for their effect on the metabolism of Formycin B in vivo. This information will help elucidate the mode of action of this drug and possibly help to determine the organism's mode of resistance to it.

Experiments were performed to determine if a membrane receptor existed for attachment of ormycin B before entry into the cell. Membranes were isolated by the methods of Dwyer, J. Protozool 27 (2) 1980, pp 176-182 and Gottlieb and Dwyer, J. Exp. Parasitol. 52, 117-128. Isotope binding assays to pure membranes were performed using H^3-Formycin B and C^{14}-adenosine. Adenosine was used as a comparison because many eucaryotic cells possess an adenosine receptor site on the membrane. The binding assay was carried out for 90 minutes with samples taken every 10 minutes. No binding of Formycin was observed. Adenosine was bound to the membrane at a concentration of .078 μM/100 μg/ml of membrane protein. Drug resistance is often accomplished by alteration or abolishment of the drug receptor site on the membrane. It appears that this mode of drug resistance to Formycin B can be ruled out. However, these experiments will be repeated in order to confirm our results.

Incorporation studies with H^3-thymidine, uridine and C^{14}-phenylalanine were performed to determine which aspect of cell metabolism was affected-DNA synthesis, RNA synthesis or protein synthesis. Cells were grown in a medium with limited purine concentration (66.6% Steiger and Black minus purine, 23.3% Sneider's medium and 10% FCS) and growth and incorporation was followed for 52 hours. As shown in Fig.6 , cells grown in the presence of 20 μM Formycin did not reproduce while those grown in the presence of 20 μM allopurinol responded very much like control cells up to 32 hours. Although incorporation was sampled every few hours, Table 2 gives only a few points.

Fig. 6

REVERSAL OF FORMYCIN B INHIBITION BY PURINES

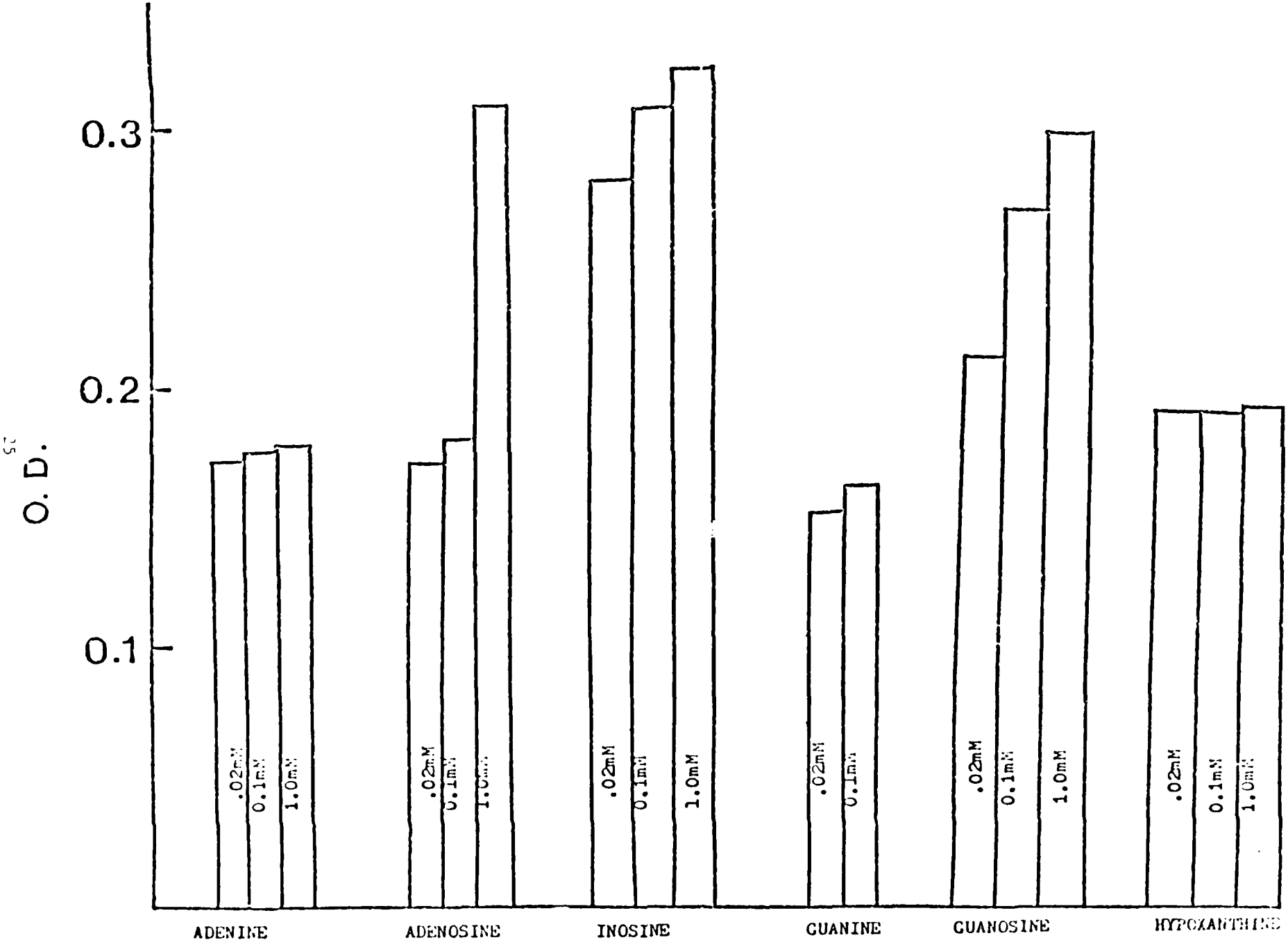


Fig. 7

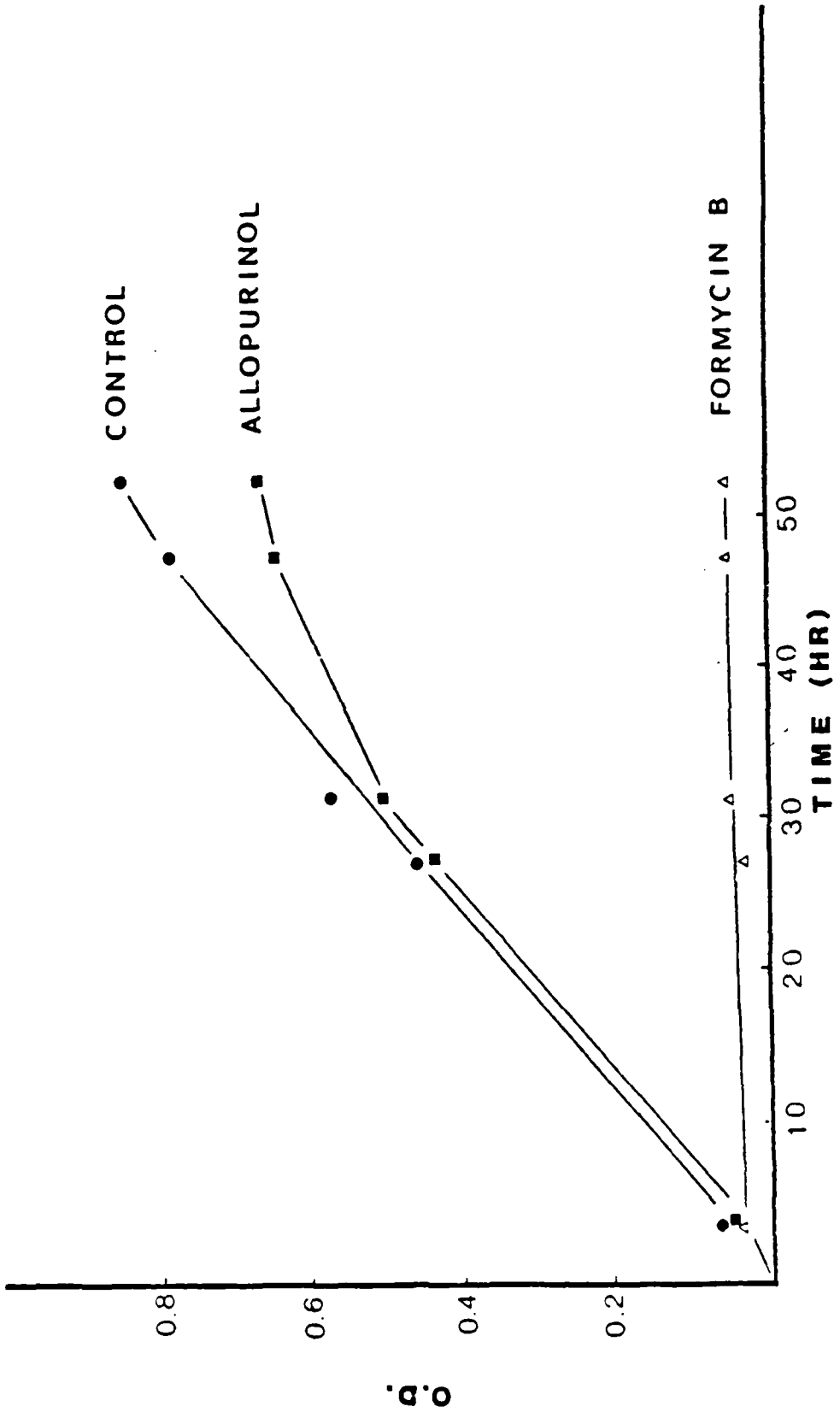

CONTROL
ALLOPURINOL
FORMYCIN B
O.D.
0.2
0.4
0.6
0.8
10
20
30
40
50
TIME (HR)

TABLE 3 Incorporation as % of Control

Cells Grown with	Time Hours	Thymidine H^3	Uridine H^3	Phenylalani C^{14}
Allopurinol	0.5	61.97%	71.4%	90.1%
Formycin B	0.5	33.5	74.8	74.5
Allopurinol	5	90.34	98	90.35
Formycin B	5	66.6	60	64.47
Allopurinol	52	100	100	84.2
Formycin B	52	5.1	9.7	7.8

Fig.7 and Table 3 clearly show that under the experimental condi Formycin B is more effective than allopurinol. In the case of Formycin cells, a decrease in incorporation of isotopes is seen within 30 minutes

Research During Last Quarter

L. braziliensis WR 227 and L. donovani WR 130 were inoculated int Steiger and Black medium with 5% heat inactivated calf serum and incorpo of ^{3}H-thymidine, ^{3}H-uridine and ^{14}C-phenylalanine into 10% TCA precipita material. (DNA, RNA, protein) was followed with time in the presence of Formycin B, allopurinol riboside and 4-aminopyrazolopyrimidine (in the c L. donovani WR 130). Initial cell concentration was 9 x 10^{6} cells/ml.

Both organisms showed the greatest sensitivity towards formycin B radioisotopes tested for incorporation into DNA, RNA & protein were dras inhibited (Tables 4 & 5). During this experiment growth inhibition monitored and at the end of 24 hr. both organisms were inhibited 74% of values in the presence of 10 μM Formycin B. Allopurinolriboside inhibi braziliensis 63.5% and L. donovani WR 130 2.77%. It appears that L. b WR227 is more sensitive to this compound. 4-Aminopyrazolopyrimidine (10 inhibited L. donovani WR 130, 37% in 24 hours.

Experiments were performed to determine if Formycin B metabolites incorporated into the mRNA and therefore interferring with translation.

Cells (250 ml) were grown in Brain Heart Infusion (Methods Sectic a concentration of 7 x 10^{8} cells/ml. They were centrifuged aseptically

TABLE: 4 Inhibition of ^{3}H-Thymidine, ^{3}H-Uridine and ^{14}C-Phenylalanine Incorporation

Compound Inhibiting at 10 µM	% Inhibition of ^{3}H-Thymidine Incorporation in *L. braziliensis* WR227 at		
	1 hr.	6 hr.	24 hr.
Formycin B	26.0	30.7	96.47
Allopurinolriboside	13.38	27.7	41.05
	% Inhibition of ^{3}H-Uridine Incorporation		
Formycin B	99	95	92.02
Allopurinolriboside	52	50	59.79
	% Inhibition of ^{14}C-Phenylalanine Incorporation		
Formycin B	100	51.6	94.7
Allopurinolriboside	51.9	11	62.5

TABLE: 5 Inhibition of ^{3}H-Thymidine, ^{3}H-Uridine and ^{14}C-Phenylalanine Incorporation in L. donovani WR 130

Compound Inhibiting at 10 uM	% Inhibition of ^{3}H-Thymidine Incorporation at 1 hr.	3.5 hr.	24 hr.
Formycin B	100	85.42	98.39
Allopurinolriboside	52.37	30.43	37.50
4-aminopyrazolopyrimidine	37.85	14.50	44.19
	% Inhibition of ^{3}H-Uridine Incorporation		
Formycin B	54.91	80.32	97.72
Allopurinolriboside	28.91	45.2	10.31
4-aminopyrazolopyrimidine	47.32	45.22	84.65
	% Inhibition of ^{14}C-Phenylalanine Incorporation		
Formycin B	0	3.2	79.17
Allopurinolriboside	7.2	0	43.2
4-aminopyrazolopyrimidine	7.4	0	81.16

resuspended into Steiger and Black (250 ml) minus purine but with 5% heat inactivated serum. To one flask (250 ml) 10 µM Formycin B was added, and incubated at 25°C for 24 hr. Control cells were not exposed to Formycin B.

Total cellular poly(A)$^+$RNA was extracted from the promastigotes by phenol-SDS extraction (Nienhuis, et al. Meth. Enzym. 30, 621-6310, (1974), and Oligo(dT)-cellulose elution (Fig 8)(Krystosek, et al, J. Biol. Chem. 250, 6077-6084, 1975). The mRNA was translated using a rabbit reticulocyte lysate (Pelham, et al., Eur. J. Biochem 67, 247-256, 1976) purchased from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Fig.9 shows the activity of the translation system between mRNA from cells exposed to Formycin B and mRNA from normal cells.

Using mRNA from cells exposed to 10 µM Formycin and comparing it to normal mRNA we have gotten from 22 - 67% inhibition of translation (not all data shown). The degree of inhibition appears to be dependent on the quantity of mRNA used in the assay system, and the fraction we use from the oligo(dT)-cellulose column. It appears that there are several species of mRNA eluting with perhaps different sensitivities to Formycin B metabolites.

To determine if nonspecific binding could occur, we heated the RNA at 65° C for 10 minutes to open the tertiary structure (routinely done to mRNA) added either 10 µM Formycin A monophosphate, Formycin A triphosphate, allopurinol riboside or 4-aminopyrazolopyrimidine and incubated 15 min. at 37°C. In all cases we got binding to mRNA which inhibited translation (Fig.10). We pre-treated the rabbit reticulolysate with these compounds (10 µM) to determine if non-specific binding would occur either on the ribosomes or with the tRNA. In all cases we got inhibition of translation (Fig.10). Using a different mRNA preparation (not exposed to drug) but eluting in a different fraction on the column, we exposed it to Formycin A mono and triphosphate 10 minutes before translation. As seen in Fig.11 , Formycin A monophosphate was more inhibitory to translation. Using the same mRNA from L. braziliensis, we then preincubated (10 min) the rabbit reticulocyte with the Formycin A mono and triphosphate. In this case Fig.11 , both compounds were equally effective in inhibiting translation. It appears from our preliminary experiments that both Formycin A mono and triphosphate can interact either with the tRNA, ribosomes or both to inhibit translation. Fig.12 shows electrophoresis of the reaction products of translation with the varying conditions as previously discussed. It appears that the inhibition is nonspecific. All major proteins appear to be synthesized under the various conditions, but they differ quantitatively. We are planning to isolate tRNA and ribosomes from L. braziliensis WR 227 and test the effect of formycin metabolites on their function.

Kumar, Krakow and Ward (Biochimica et Biophysica Acta, 477 (1977) 112-124) have reported that analogues such as formycin triphosphate and tubercidin triphosphate can be incorporated into complementary polyribonucleotides but not as efficiently as ATP. The synthesis of RNA is the net result of a sequence of reactions involving initiation, translocation and elongation. The inability of an analogue to replace efficiently ATP may be a consequence of impairment of

Fig. 8

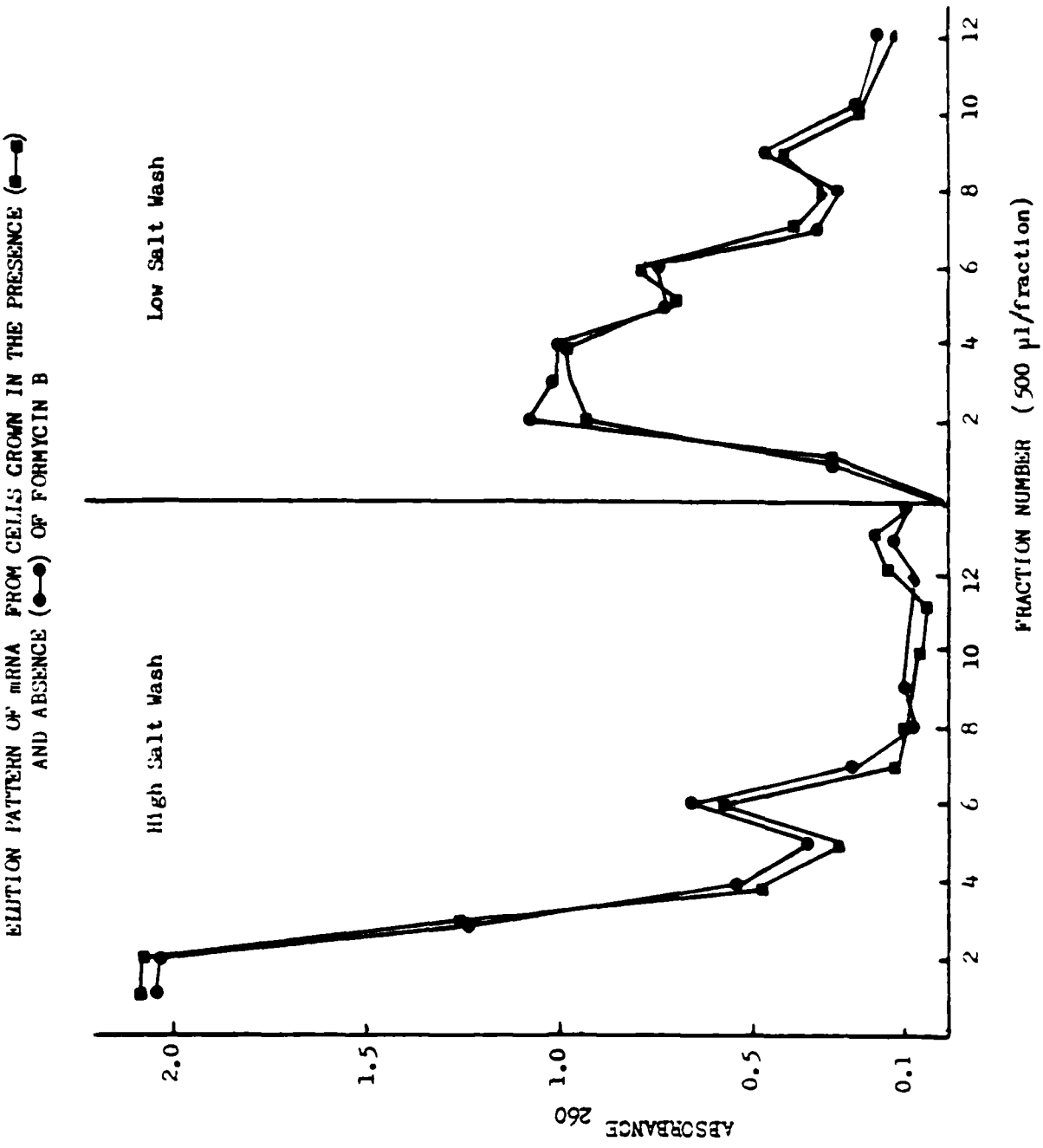
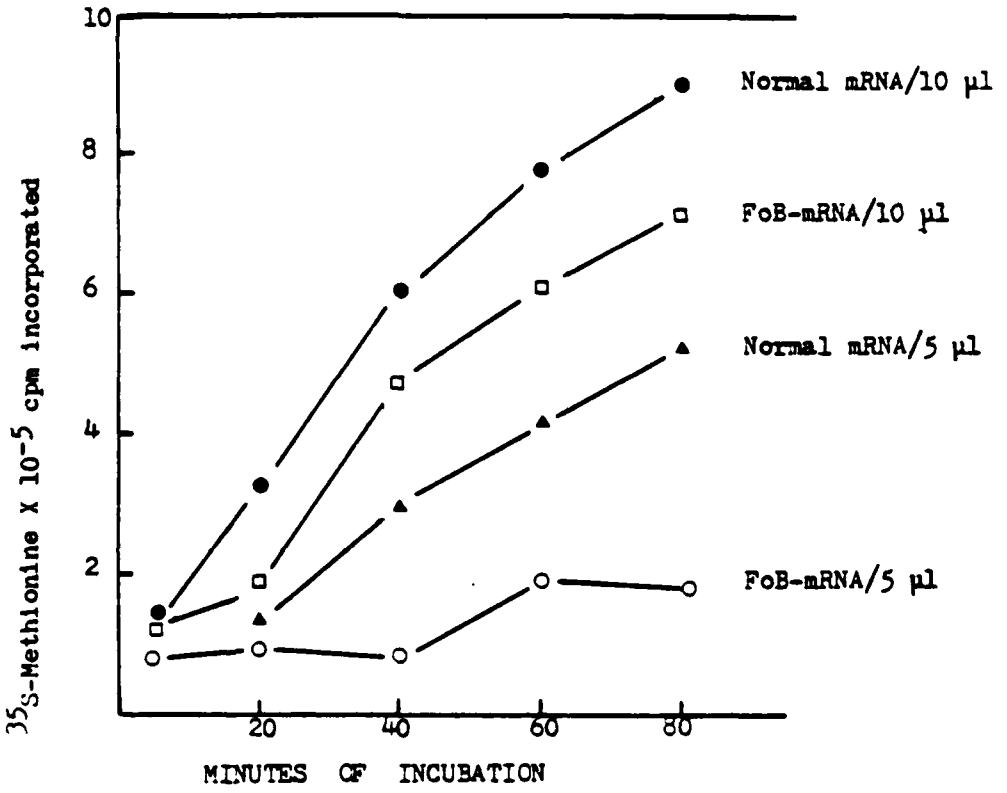
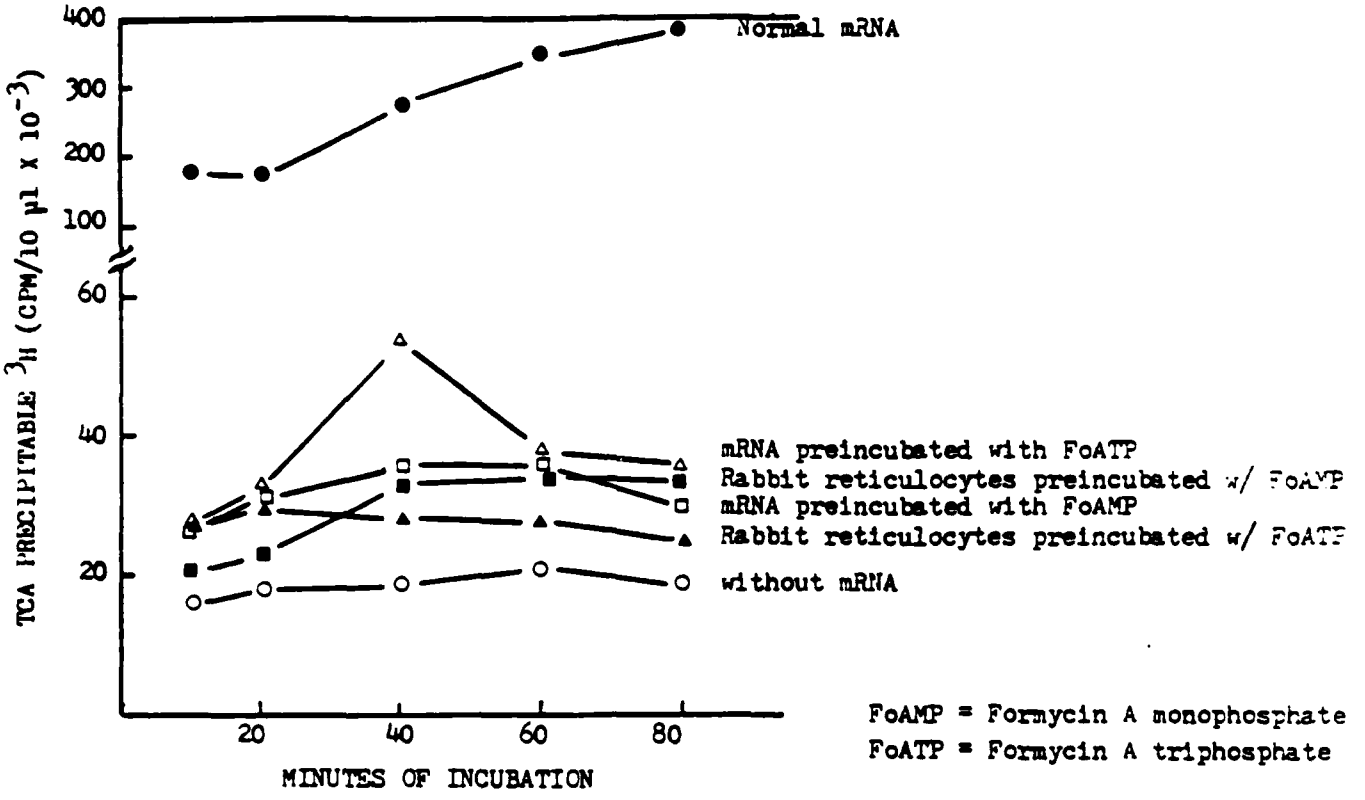

ELUTION PATTERN OF mRNA FROM CELLS GROWN IN THE PRESENCE (■—■) AND ABSENCE (●—●) OF FORMYCIN B
High Salt Wash
Low Salt Wash
ABSORBANCE 260
2.0
1.5
1.0
0.5
0.1
2
4
6
8
10
12
2
4
6
8
10
12
FRACTION NUMBER (500 µl/fraction)

Fig. 9

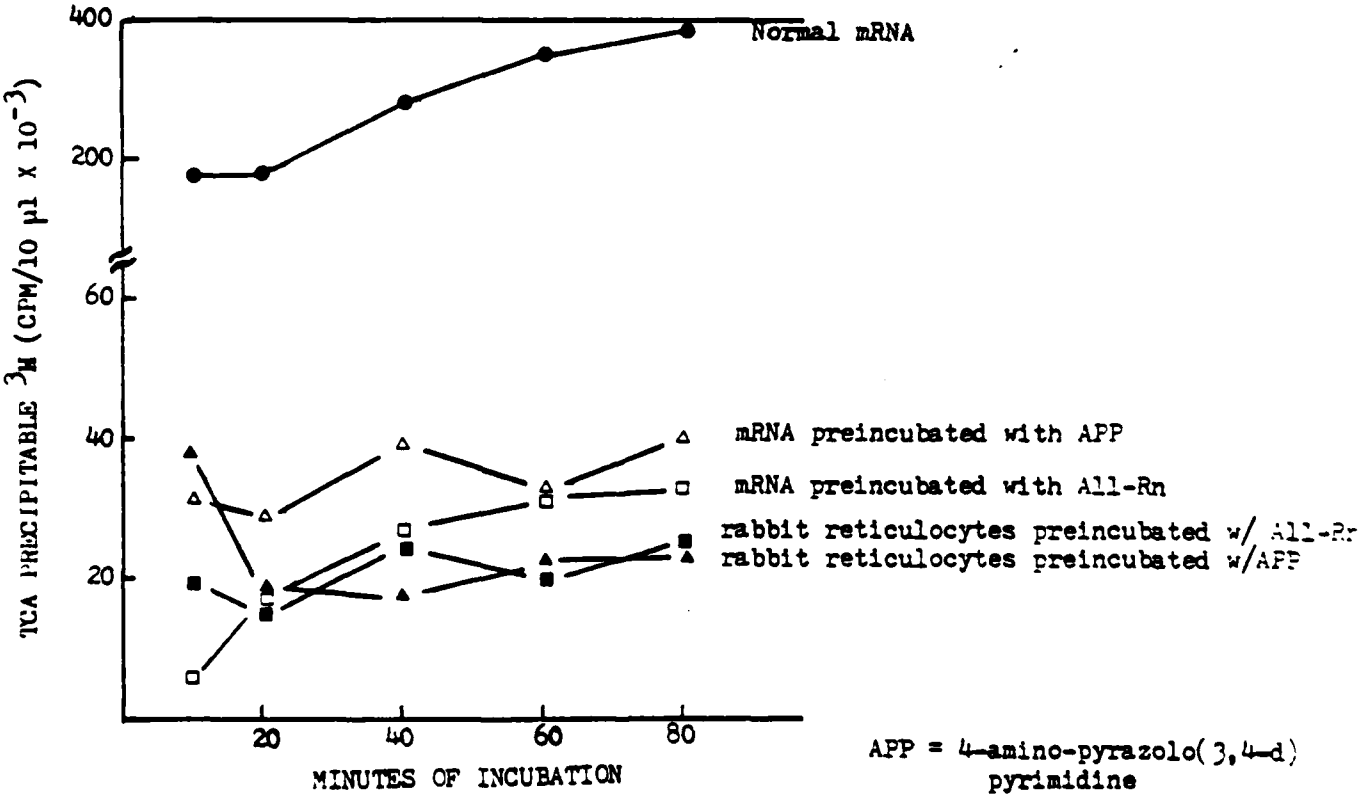


messenger RNA obtained from cells grown in the presence (FoB) or absence (Normal) of Formycin B

Fig. 10



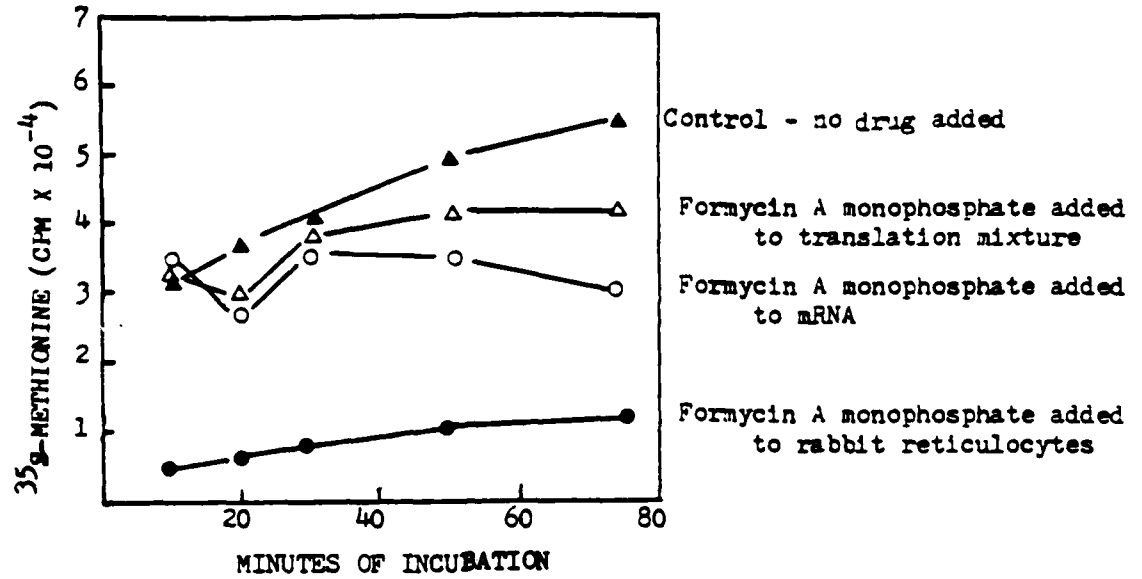
FoAMP = Formycin A monophosphate
FoATP = Formycin A triphosphate



APP = 4-amino-pyrazolo(3,4-d) pyrimidine

All-Rn = allopurinol ritonucleoside

Fig. 11


7
6
5
4
3
2
1
20
40
60
80
^{35}S-METHIONINE (CPM X 10^{-4})
MINUTES OF INCUBATION
Control - no drug added
Formycin A monophosphate added to translation mixture
Formycin A monophosphate added to mRNA
Formycin A monophosphate added to rabbit reticulocytes

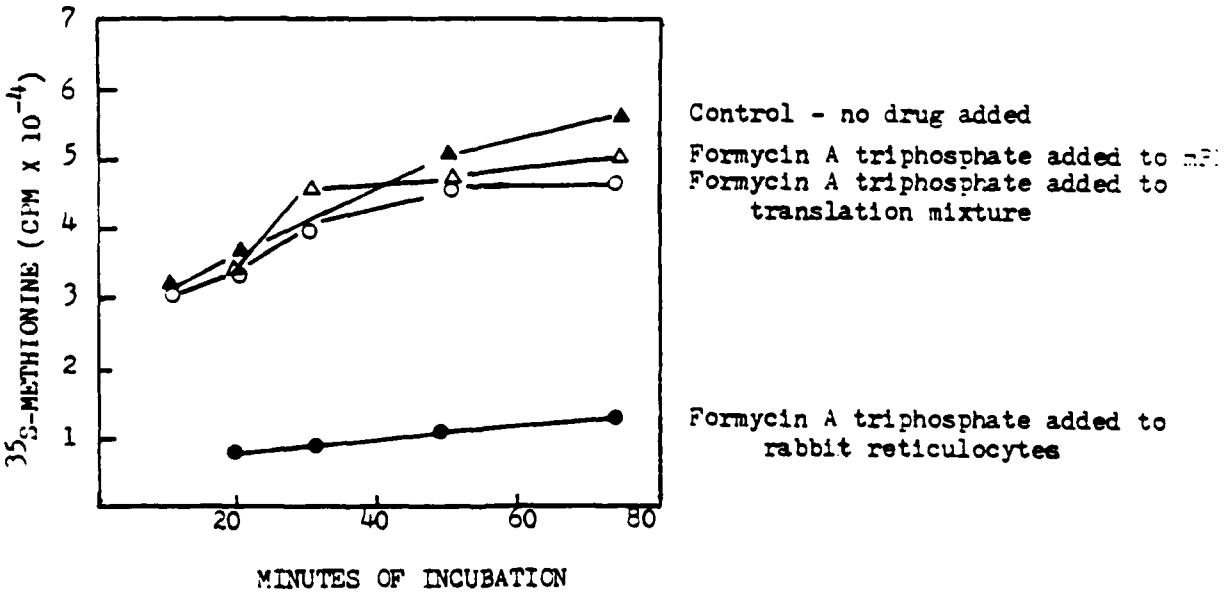
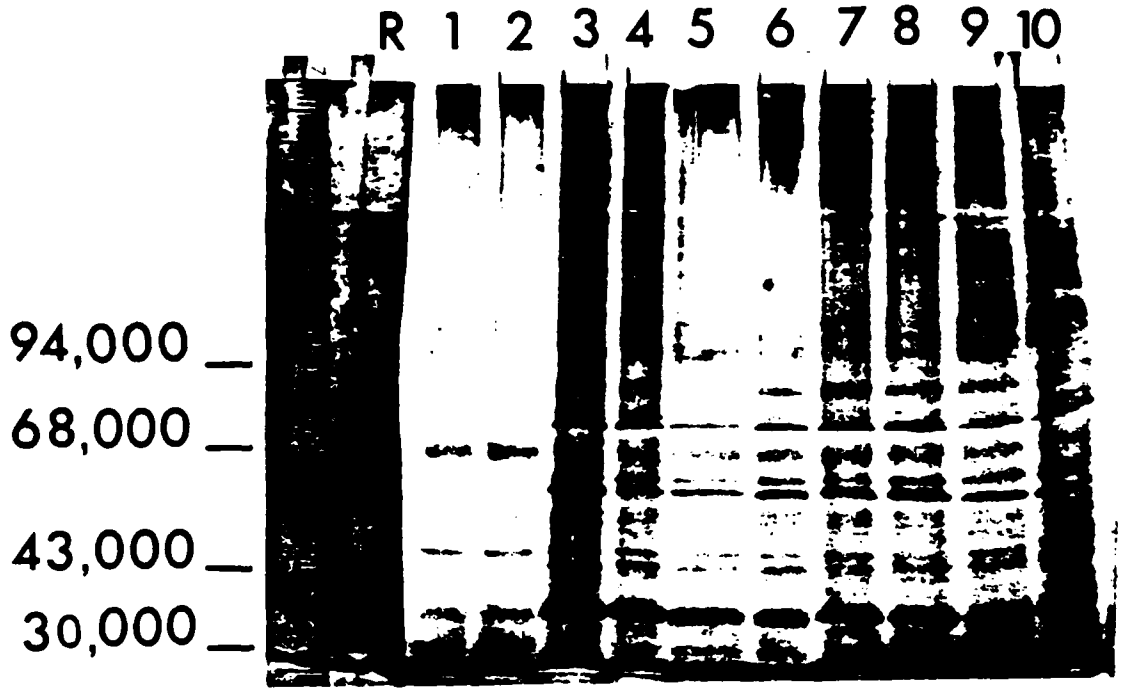

7
6
5
4
3
2
1
20
40
60
80
^{35}S-METHIONINE (CPM X 10^{-4})
MINUTES OF INCUBATION
Control - no drug added
Formycin A triphosphate added to mRNA
Formycin A triphosphate added to translation mixture
Formycin A triphosphate added to rabbit reticulocytes

Fig. 12

Gel Electrophoresis of mRNA Translation Products



Conditions for Electrophoresis

The translation products were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis (Laemmli, 1970). The electrophoresis was carried out on a 7.5% acrylamide - 0.13% bis-acrylamide gel in 0.05 MTris, 0.38 M glycine pH 8.8, 0.1% SDS buffer at 120 volts for two hours. After the run, gel was stained by silver nitrate staining procedure of BIORAD CO.

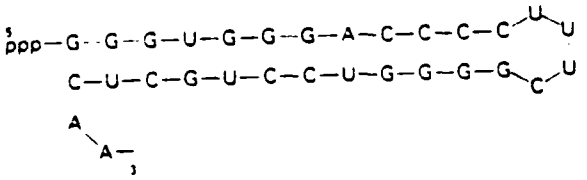
Laemmli, U.K. (1970) *Nature* 227, 680-685.

one or more steps in the reaction. Each step should be assayed separately in order to determine the extent to which an analogue can replace ATP at each step. We will use the method of Kumar et al as cited above to determine how the metabolites of formycin, allopurinol and 4-aminopyrazolopyrimidine function for (a) initiation (b) single-step addition and (c) polymerization.

What sets tRNA apart from the other cellular nucleic acids is the high frequency and large variety of modified nucleosides contained in tRNA molecules. It is clear that to date very few definite roles for modified nucleosides in tRNA have been found. However, there is a great deal of information as to the relative change in amounts of modified nucleosides during such cellular phenomena as differentiation, senescence, neoplasia, chemical carcinogenesis, and viral transformation (Littauer, et al. Annu. Rev. Biochem. 42, (1973)) 439-470.

Since tRNA naturally contains modified nucleosides, it appears possible that purine analogues are more easily incorporated into its structure.

It is well known that since RNA is single stranded it can fold back on itself or form hydrogen bonds with purines and pyrimidines which modify its function.



RNA can fold back on itself to form double-helical regions.

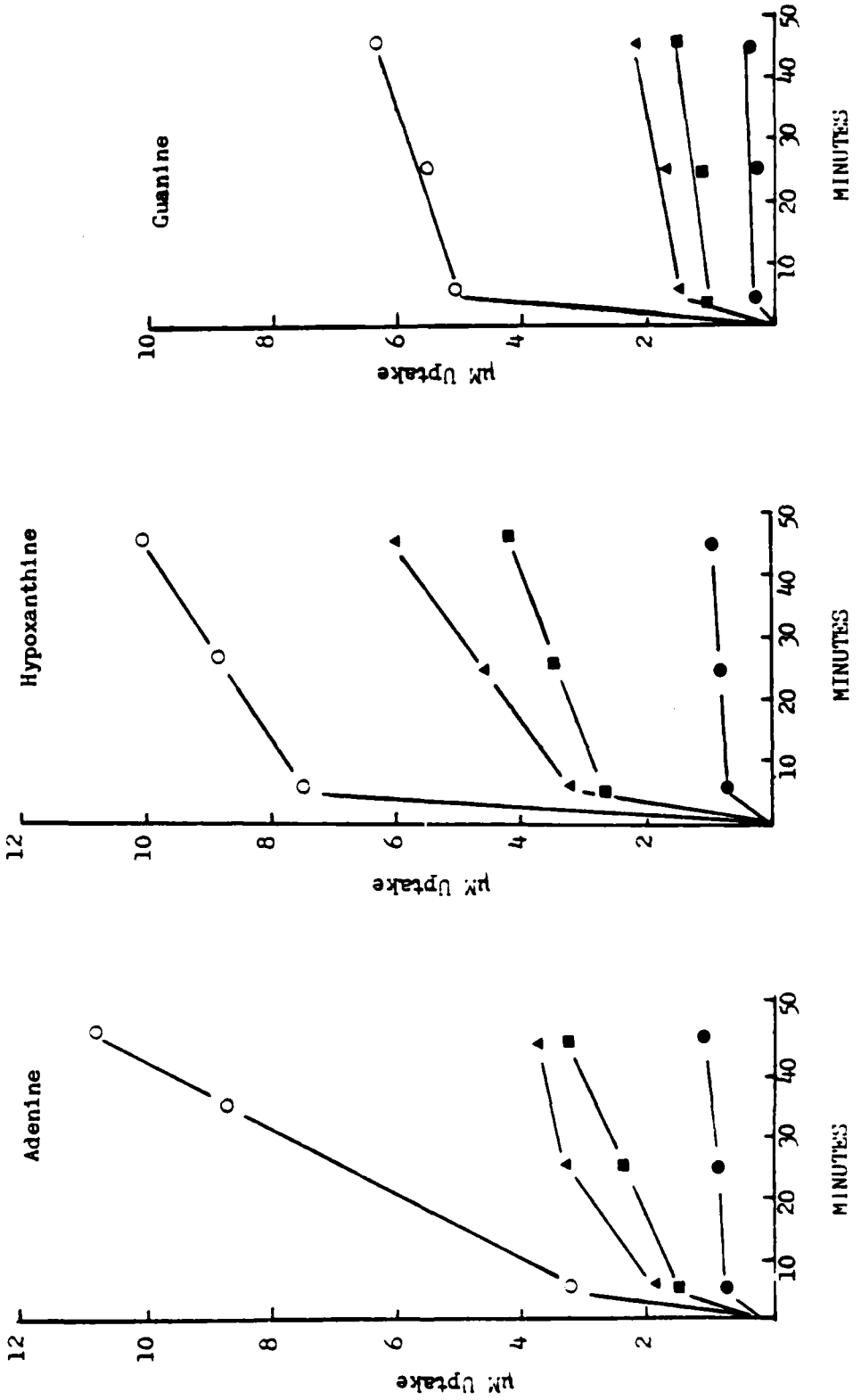
We plan to investigate further the nonspecific binding of formycin and its metabolites to RNA during various conditions, such as pH, ionic strength of environment, temperature and the presence of natural purines.

Studies on Purine and Pyrimidine Uptake with L. braziliensis WR 227

Uptake experiments were performed as described in Methods, using 10 uCi radiolabeled purine bases and nucleosides at various concentrations. This experiment was performed to determine if increasing concentrations might inhibit the uptake process (transport and accumulation). If the trapping of the bases or nucleosides were impaired (via phosphoribosyltransferases or kinases) one would see a leaking out of the substrate with time. We found in all cases that increasing the substrate 1 - 50 uM improved transport and accumulation up to 45 minutes.

Uptake of nucleosides was tested for competitive interactions. Labeled nucleoside being tested was at 5 uM and its nucleoside antagonist at 50 uM. It appears (data not shown) that inosine and guanosine share a common transport protein and/or trapping mechanism (phosphorylation mechanism). Assay times

Fig. 13


UPTAKE OF ^{14}C-PURINE BASES AS A FUNCTION OF INCREASING CONCENTRATION
Adenine
Hypoxanthine
Guanine
μM Uptake
MINUTES
50 μM
10 μM
5 μM
1 μM

were from 4 - 30 minutes. Adenosine was antagonized by guanosine, but guanosine was only slightly antagonized by adenosine. Thus it appears that there are at least two distinct loci for transport of nucleosides. One with a high affinity for adenosine, and one which transports inosine and guanosine. A similar observation involving uptake of adenosine and inosine was observed by Hansen et al. (Parasitology, 83, 271-282, 1982).

Since the main objective of this experiment was to observe accumulation with time, no attempt was made to measure transport as distinct from diffusion.

Several analogues were tested to determine if they antagonized the uptake of radiolabeled adenosine and inosine. Nucleoside concentration was 30 µM and the analogue at 150 µM. As shown in Table 6 adenosine uptake was antagonized to a much greater extent than inosine uptake. Inosine uptake was most affected by 6-mercaptopurineriboside which did not affect adenosine uptake significantly. This adds further proof to the fact that there must be separate loci for transport and/or accumulation of inosine and adenosine.

Growth Experiments

Several purine and pyrimidine analogues were screened for growth inhibitory properties. L. braziliensis WR 227 and L. donovani WR 130 were grown in 5 ml/tube of Steiger and Black minus adenosine but supplemented with 0.08 mM hypoxanthine and 10% heat inactivated fetal calf serum. Growth was followed for several days. Table 7, 8 shows the response of these organisms to the various analogues. Major differences which appeared between the two organisms were the growth stimulation of various compounds for L. braziliensis 227 and the observation that L. donovani was slightly inhibited by 6-methoxy-purine while L. braziliensis was not. From the growth stimulatory data, one might infer that L. braziliensis can transport these analogues better and/or is better able to catabolize them to a natural purine or pyrimidine. In this experiment cells were inoculated to 4×10^6 cells/ml and at the end of 72 hours, L. donovani control was 4.2×10^7 cells/ml and L. braziliensis grew to 1.6×10^7 cells/ml. Growth was monitored measuring O.D. using a Spectronic 21 at a wavelength of 660 nm.

TABLE: 6 The Effect of Analogues on ^{3}H-Purine Nucleoside Uptake

Analogue being tested 0.15 mM	In the Presence of 0.03 mM H^{3}- Adenosine		Inosine	
	% Inhibition			
	4 min.	30 min.	4 min.	30 min.
Allopurinol riboside	0.65	62.3	36.6	17.1
Formycin A	100	15.5	20.2	10.7
Allopurinol	0	0	100	20.7
Formycin B	0	3.63	59.5	28.1
Hypoxanthine-9β-Darabinofuranoside	0	18.5	75.1	32.5
6-methylpurine	100	52.6	48.2	11.9
6-methyloxypurineriboside	100	75.9	0	0
6-mercaptopurineriboside	100	2.53	93.5	66.2
adenosine,N^{6}-cyclohexyl	100	66.0	0	0
8-phenyltheophylline	100	22.2	0	0
Uridine	100	54.8	72.4	28.1

TABLE:7 Compounds Found to Inhibit Growth

Compound	% Inhibition at 72 hr.	
	L. braziliensis WR 227	*L. donovani* WR130
4-amino-pyrazolo(3,4-d)pyrimidine (0.5 mM)	91.5	94.1
2-mercaptopyrimidine (0.5 mM)	91.5	0
5-fluorouracil (0.5 mM)	81.5	91.2
4-mercapto-2H-pyrazolo[3,4-d]pyrimidine (0.2 mM not as soluble as the others)	81.5	41.3
6-methoxypurine-riboside	0	17.8

TABLE:8 Compounds Found to be Growth Stimulators

Compound	% Stimulation at 72 hrs.	
	L. braziliensis WR 227	L. donovani WR
6-methoxypurine-riboside 0.5 mM	23.8	0
5-diazouridine 0.5 mM	28.1	1.2
5,6-diamino-2,4-dihydroxypyrimidine 0.25 mM	28.0	10.3
5-p-nitrobenzyl-6-thioinosine 0.25 mM	68.5	0.58
6-(4-nitrobenzyl)thioinosine 0.2 mM	53.8	0

END

FILMED

2-85

DTIC